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Laboratory Induction and Clinical Occurrence of Combined Clindamycin and Erythromycin Resistance in Corynebacterium acnes

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***Corynebacterium acnes* strains cross-resistant to clindamycin and erythromycin were observed following long-term selection or mutagenic treatment in the laboratory. Similar strains were found among clinical isolates from patients using clindamycin or erythromycin topically in the treatment of acne vulgaris. Clindamycin resistance was never observed in the absence of resistance to macrolides or other lincosaminides. It is suggested that this resistance may result from an alteration of the 50S ribosomal subunit.**

Clindamycin is a lincosaminide antibiotic which has been shown to be effective in the treatment of acne vulgaris, probably through its antibacterial action against *Corynebacterium acnes** [1]. The effectiveness of clindamycin might be diminished if *C. acnes* developed resistance to it.

We began a search for clindamycin-resistant strains of *C. acnes* to ascertain their prevalence and clinical importance and, if possible, to study the mechanism of their resistance. Four different approaches were taken in the search for resistant strains: (1) Exposing a wild type *C. acnes* strain to clindamycin to see how readily spontaneous resistance occurs in the laboratory, (2) applying stepwise selective pressure by transferring a wild type strain into increasing levels of clindamycin, (3) exposing a growing culture to a mutagen to induce resistant mutants, and (4) isolating *C. acnes* strains from patients who had been treating themselves topically with clindamycin and searching for resistant strains among these isolates.

Sugar fermentation, gel liquefaction, production of indole and agglutination by specific antisera were used to characterize the strains used in this study. Strains were also tested for sensitivity to ampicillin, bacitracin, kanamycin, neomycin, lincomycin, erythromycin, oleandomycin, streptomycin, penicillin, and tetracycline.

MATERIALS AND METHODS

Isolation of Strains

The wild type strain and the clinical isolates were obtained from open comedones. The area to be sampled was wiped with 70% isopropyl alcohol and the comedo expressed with a Schamberg comedo extractor. The contents were emulsified in sterile Triton-X phosphate buffer (0.067 M Na_2HPO_4, 0.067 M KH_2PO_4, 0.05% Triton-X100, pH 7.9) diluted, and streaked onto Brain Heart Infusion agar (BHIA) (Difco) plates. After incubation anaerobically for 3–4 days at 37°C, presumptive *C. acnes* colonies were identified by their small size (<1 mm) and beige to pinkish-beige color. Verification was by Gram stain. Cultures were maintained in thioglycollate broth tubes (Difco) with 1% Tween 80, incubated aerobically at 37°C and transferred every 4–5 days.

Antibiotic Tests

Antibiotic sensitivity tests were made by inoculating 0.1 ml of a growing culture into sterile thioglycollate broth tubes containing the desired concentration of antibiotic. Tubes were inspected after 2 and 7 days for signs of growth. Clindamycin sensitivity was also tested on BHIA plates by adding the desired concentration of the antibiotic to the agar before autoclaving (clindamycin is stable to autoclaving at neutral pH) and spreading 0.1 ml of a growing culture onto the plates. Antibiotic disc tests were done by spreading a lawn of cells (0.1 ml) onto a BHIA plate and placing an antibiotic disc in the center of the plate. All plates were incubated in anaerobic jars (Gas Pak-Baltimore Biological Laboratories) for 4 days. Discs that were prepared by us utilized blank paper discs (QBBL-Becton Dickinson & Co., Cockeysville, Maryland) 0.25 inches in diameter which were dipped into different concentrations of clindamycin HCL or erythromycin base. The solvent for the antibiotics was 30% M-pyrol in 70% isopropyl alcohol. The discs were air dried before use. All antibiotic solutions were prepared within 2 days of use in the experiments.

Selection Experiment

A tube containing 10 ml of thioglycollate broth plus 0.01 μg/ml clindamycin was inoculated with 0.1 ml of a growing wild type culture. Cells which grew in the 0.01 μg/ml tube served as inoculum (0.1 ml) for tubes containg higher levels of clindamycin (0.025, 0.04 and 0.05 μg/ml). Cells from the last tube to show growth were then transferred into tubes containing still higher levels of clindamycin. Thus, resistance was selected for by exposing the wild type strain to gradually increasing clindamycin concentrations.

Mutagenesis

N-methyl-N′-nitro-N-nitrosoguanidine (NTG) was added to a wild type culture growing exponentially in 3 ml of thioglycollate broth. In various experiments NTG concentrations from 30–300 μg/ml and exposure times of 15 to 40 min were used. A control tube to which no NTG was added was included in each experiment. After treatment the cells were centrifuged, washed with 10 ml of sterile TM buffer (prepared according to [2] with bis-tris-propane (Sigma) substituted for 2-amino-2-(hydroxymethyl)-1,3-propanediol), and resuspended to 10 ml of fresh thioglycollate broth. Viable cell counts were done on BHIA plates immediately after resuspension in broth to ascertain the percentage of cells killed. Treated cells were then incubated for 1–2 days before being spread (0.1 ml) on clindamycin-containing BHIA plates to detect resistant mutants.

Biochemical Tests

Carbohydrate utilization tests were done in thioglycollate broth containing 0.05% bromophenol blue but without dextrose or resazurin. Glycerol, D-glucose, D-mannose, D-galactose, lactose, maltose, glycogen, D-fructose and sucrose were added to the tubes in a final concentration of 1%. Fermentation was detected by the development of a yellow color in the tube.

Gelatin liquefaction was determined by Frazier's precipitation method [3]. The center of a gelatin agar plate was inoculated with 0.05

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Abbreviations:
NTG—N-methyl-N′-nitro-N-nitrosoguanidine
BHIA—Brain heart infusion agar

* Whether the organism should be called *Corynebacterium acnes* or *Propionibacterium acnes* is still unresolved so we will refer to it as *C. acnes* in this manuscript.

ml of a suspension of *C. acnes*. After 7 days of anaerobic incubation, the plate was flooded with acidic $HgCl_2$.

Indole production was tested in tryptose yeast extract glucose thioglycollate broth [4]. Indole was extracted from the medium with toluene and detected with indole reagent.

Serological Tests

Commercial *C. acnes* antisera numbers 554 and 605 (Difco) were used to agglutinate washed cell suspensions in slide agglutination tests. Agglutination in one or both antisera in the absence of agglutination in a saline control constituted a positive *C. acnes* identification.

All clinical samples from acne patients were comedones taken with a Schamberg comedo extractor and processed as reported in a previous communication [1]. The clinical samples from patients 1–10 in Table I were tested on agar into which clindamycin HCl was incorporated in different concentrations. Subsequent isolates were tested as effectively and more conveniently by observing the lowest of 5 concentrations of clindamycin discs causing clear inhibition of growth in the disc inhibition test. The solutions into which the discs were immersed before air drying were 1.0, 0.1, 0.01, 0.001 and 0.0001 percent clindamycin.

RESULTS

The minimum concentration of clindamycin completely inhibiting growth of wild type *C. acnes* is about 0.1 μg/ml in thioglycollate broth and about 0.02 μg/ml in BHIA plates. Resistance of individual wild type cells varied, with some cells sensitive to much lower levels of the antibiotic. No wild type cells were ever found to be resistant to clindamycin at 1 μg/ml in broth.

The selection experiment produced a strain (S1) resistant to more than 100 times the concentration of clindamycin completely inhibitory to the wild type. This level of resistance was very slow to develop, however. Early in the experiment resistance increased by only about 0.05 μg/ml at each transfer. As the culture became more resistant it was able to tolerate greater increases in clindamycin concentration. Twelve transfers were required to produce S1.

The mutagenesis experiment was performed to see if resistance could be developed by mutation in a single step. Mutagenesis was attempted in 12 experiments using several concentrations of nitrosoguanidine for varying exposure times. Only one resistant mutant (M1) was obtained in these experiments. M1 came from a *C. acnes* culture which had been treated with 100 μg/ml of nitrosoguanidine for 15 min. This treatment killed 26% of the cells in the culture. M1 proved to be resistant to high levels of both clindamycin and erythromycin, but at high clindamycin levels it grew somewhat more slowly than S1. Similar erythromycin levels did not inhibit its growth.

TABLE I. *Sensitivity of C. acnes isolated from comedones of patients using 1% topical clindamycin lotion*

Patient No.	Sex	Time on clindamycin lotion	Minimal inhibitory clindamycin concentration	
			In BHIA[a] agar (μg/ml)	In discs (% solution)[b]
1	F	2 mo	0.1	
2	F	2 yr	NR[c]	
3 (R1)	F	6 mo	>20.0	
4	F	3 yr	NR	
5 (R2)	F	2 yr	>20.0	
6	F	1 yr	0.01	
7	F	9 mo	0.15	
8	F	4 mo	0.05	
9	M	4 mo	0.15	
10	F	2 mo	NR	
11	F	4 mo		NR
12	F	12 mo		10
13	F	10 mo		0.001
14	M	4 mo		NR
15	F	4 mo		NR
16	F	10 mo		10
17	M	5 mo		0.001
18	F	1 yr		NR
19	F	2 mo		0.01
20	M	Many mo		0.01
21	F	4 mo		0.01
22	M	4 mo		NR

[a] BHIA = brain heart infusion agar.
[b] Blank discs immersed in solution and then air dried before use.
[c] NR = No *C. acnes* recovered from comedones.

TABLE II. *Sensitivity of C. acnes isolated from comedones of patients using 1% topical erythromycin lotion*

Patient No.	Sex	Time on erythromycin lotion	Minimum inhibitory concentration of discs[a]	
			Erythromycin	Clindamycin
1	F	8 weeks	1.0	1.0
2	F	8 weeks	0.01	0.001
3	M	8 weeks	0.001	0.001
4	F	8 weeks	0.01	0.01
5	M	8 weeks	1.0	1.0
6	F	8 weeks	0.001	0.001
7	F	8 weeks	0.01	0.01
8	M	8 weeks	0.01	0.001
9	M	8 weeks	1.0	1.0
10	F	8 weeks	0.001	0.001
11	F	8 weeks	1.0	1.0
12	F	8 weeks	0.01	0.01
13	M	8 weeks	0.01	0.001
14	F	8 weeks	0.001	0.001
15	F	8 weeks	0.001	0.001
16	M	8 weeks	0.01	0.001
17	F	8 weeks	NR[b]	NR
18	F	8 weeks	0.01	0.001
19	F	8 weeks	NR	NR
20	M	8 weeks	0.001	0.001
21	F	8 weeks	0.001	0.001

[a] Blank discs immersed in solution and air dried before use. Antibiotic discs made from solutions of 5 different concentrations (1.0, 0.1, 0.01, 0.001 and 0.0001 percent) of antibiotic.
[b] NR = No *C. acnes* recovered from comedones.

The third method of obtaining clindamycin resistant *C. acnes* was by isolation from comedo samples taken from patients treating themselves topically with clindamycin for over 4 months. Of 22 patients tested, 5 gave rise to strains showing resistance to high levels of clindamycin and one had a strain which possessed intermediate resistance (Table I). Comedo samples were also taken from 21 patients on topical 1% erythromycin lotion for acne vulgaris for 8 weeks. From 4 of the 21 subjects *C. acnes* resistant to erythromycin was isolated. The level of resistance observed allowed growth on agar plates next to discs saturated with erythromycin at 10 mg/ml. All these organisms resistant to erythromycin were also resistant to comparable levels of clindamycin (Table II).

Representative strains from each type of experiment (selection, mutation and clinical isolation) were tested for biochemical properties, specific antiserum agglutination and antibiotic sensitivity. Results are shown in Table III and Table IV. The strains were identical biochemically and only slight differences were noted in antibiotic sensitivity. On the basis of the biochemical tests, all strains used in this study seem to be of the type designated *C. acnes* Group I (*Propionibacterium acnes*) by Voss [5].

DISCUSSION

Clindamycin, lincomycin and the macrolide antibiotics tested in this study act by binding to the 50S ribosomal subunit and inhibiting protein synthesis. By analogy with other inhibitors, resistance to such antibiotics could be due to (1) synthesis of an enzyme capable of inactivating the antibiotic, (2) prevention of access of the antibiotic to the ribosome, or (3) modification of the ribosomes so they become insensitive to the antibiotic. The data derived from this study are not sufficient to define the type of resistance found in *C. acnes*. However, the results

TABLE III. *Characterization of wild type and resistant strains of C. acnes*

Strain	Source	Antiserum agglutination		Biochemical Tests			Antibiotic Disc[b] Sensitivities		
		554	605	Sugars fermented[a]	Indole production	Gelatin liquefaction	Lincomycin	(mm of inhibition) Erythromycin	Oleandomycin
Wild type	Comedo (normal)[c]	+	+	Glu, Man, Gly, Fru, Gal	+	+	18	29	19
S1	Selection	+	+	Glu, Man, Gly, Fru, Gal	+	+	0	0	0
M1	Mutation	+	+	Glu, Man, Gly, Fru, Gal	+	+	0	0	0
R1	Comedo (patient)[d]	+	+	Glu, Man, Gly, Fru, Gal	+	+	0	0	0
R2	Comedo (patient)[d]	+	+	Glu, Man, Gly, Fru, Gal	+	+	0	0	0

[a] Abbreviations: Glu, D-Glucose; Man, D-Mannose; Gly, Glycerol; Fru, D-Fructose; Gal, D-Galactose. Fermentation tests required 4–12 days to become positive and were considered negative after 14 days.
[b] Discs contained 2 μg of the antibiotic.
[c] Normal subject—no topical or systemic antibiotics.
[d] Patients with acne treated for over 6 mo with topical clindamycin 1% solution.

TABLE IV. *Antibiotic sensitivity of wild type and resistant strains of C. acnes*

Strain	Source	Minimal inhibitory concentration[a] in thioglycollate broth								
		Clindamycin	Erythromycin	Tetracycline	Bacitracin	Ampicillin	Penicillin	Streptomycin	Kanamycin	Neomycin
Wild type	Comedo	0.10	0.125	2–3	0.5–1	0.3–0.5	0.1–0.3	50–70	100–200	100–200
S1	Selection	>20	>20	1–2	0.3–0.5	0.3–0.5	0.1–0.2	25–50	100–200	100–200
M1	Mutation	>20	>20	1–2	0.1–0.3	0.2–0.4	0.1–0.3	25–60	100–200	50–100
R1	Comedo (patient)	>20	>20	1–2	0.5–1.0	0.3–0.5	0.5–0.7	50–75	100–200	50–100
R2	Comedo (patient)	>20	>20	5	0.3–0.5	0.5–1.0	0.7–1.0	50–75	100–200	50–100

[a] Concentrations (μg/ml except for bacitracin and penicillin which are in units/ml) showing little or no growth after 7 days.

described, and some additional experiments which were performed, provide some clues to the probable mechanism.

In an attempt to detect the presence of an enzyme capable of inactivating clindamycin, resistant strains were streaked on one half of clindamycin-containing BHIA plates and the sensitive wild type strain was streaked on the other half. Concentrations of clindamycin near the minimal inhibitory level were chosen. If an inactivating enzyme not firmly bound to the cell were being produced, it might diffuse out from the resistant organisms and permit growth of the sensitive strain. However, none of the plates showed growth of sensitive organisms adjacent to the resistant strain.

A second attempt to detect the presence of an inactivating enzyme involved inoculating sensitive cells into clindamycin-containing thioglycollate broth which had previously supported the growth of resistant organisms. The spent medium was sterilized by filtration and mixed with fresh sterile thioglycollate broth before inoculation with the sensitive strain. Sensitive organisms could grow in this medium only if the clindamycin had been inactivated by the growth of resistant cells. No indication of clindamycin inactivation was obtained.

Resistance due to prevention of access of the antibiotic to the ribosome is possible, but seems unlikely because of the demonstrated cross-resistance to 2 different classes of antibiotics, lincosaminides and macrolides. It seems improbable that transport of 2 such dissimilar compounds to the interior of the cell should be impeded without affecting passage of other antibotics and compounds necessary for cell growth.

A more likely mechanism of resistance that might account for macrolide and lincosaminide cross-resistance involves a change in some component of the 50S ribosomal subunit. Weisblum and Demohn [6] described a ribosomal modification in *Staphylococcus aureus* which gives resistance to macrolides and lincosaminides but not to other 50S ribosomal inhibitors or to antibiotics acting elsewhere. This mechanism involves an alteration of the 23S rRNA induced by low levels of erythromycin. Presumably this change in the 23S rRNA prevents lincosaminides and macrolides from binding to the 50S ribosomal subunit but allows the ribosome to remain functional. The fact that other 50S ribosomal inhibitors are still effective suggests that lincosaminides and macrolides share at least part of a common binding site on the 50S subunit.

In a search for induced resistance in *C. acnes* similar to that described in *S. aures* [7], cells were grown in tubes containing subinhibitory levels of erythromycin and subsequently tested for resistance to higher levels of erythromycin or to clindamycin. Antibiotic-containing discs were also used to test for induction. Erythromycin discs were placed near discs containing other macrolides or lincosaminides. A decreased radius of inhibition by lincosaminides or other macrolides in the areas near the erythromycin disc would suggest induced resistance [6, 7]. No evidence for induction was found with either of these methods.

Though the mechanism of clindamycin resistance in *C. acnes* is probably not an inducible (or constitutive) alteration in the 23S rRNA, a change in the 50S ribosomal subunit could still account for the cross-resistance observed in this study. Tanaka et al [8] described an erythromycin resistant mutant of *Escherichia coli* having an altered 50S ribosomal protein and a reduced ability to bind erythromycin. Teraoka, Tamaki, and Tanaka [9] found this erythromycin-resistant strain to be less active in protein synthesis than the wild type. Tanaka et al [10] demonstrated that resistance to erythromycin was always accompanied by cross-resistance to other macrolide antibiotics.

The possibility of an altered ribosomal protein in *C. acnes* conferring resistance to macrolide and lincosaminide antibiotics is compatible with all the data obtained in this study. If indeed lincosaminide and macrolide antibiotics share a common or overlapping binding sites, then cross-resistance to the clindamycin-resistant variants could be due to a mutant ribosomal

protein. Resistant strains, especially those developed through selection in the laboratory, were found to be somewhat slower growing in antibiotic-free media than sensitive strains. Clindamycin-resistant strains induced in the laboratory were also more sensitive to some nonmacrolide antibiotics than wild type strains (Table IV). Both of these observations could be due to an impairment in the protein synthesizing efficiency of resistant ribosomes.

The fact that the single-step resistant mutant M1 showed a growth rate inhibition by clindamycin could be explained by a slight change in ribosomal protein structure sufficient to lessen but not completely destroy the affinity of clindamycin for the ribosomal binding site. Perhaps more than a single amino acid change is necessary to alter the site enough to prevent clindamycin binding entirely. Interestingly, the rate of growth of strain M1 is normal in the presence of erythromycin and other macrolides. This may indicate that the binding sites for clindamycin (lincosaminides) and erythromycin (macrolides) are not identical.

In the clinical use of topical clindamycin and erythromycin there is evidence for resistant *C. acnes* organisms developing in about 20% of the subjects. When organisms become resistant to 1 of these 2 antibiotics they develop resistance to the other antibiotic at the same time. We have preliminary evidence that normally sensitive strains of *C. acnes* return and take over the comedones if topical clindamycin or erythromycin lotions are stopped for 1–2 mo.

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